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Mutual effects of MinD–membrane interaction: I. Changes in the membrane properties induced by MinD binding

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ABSTRACT

In *Escherichia coli* and other bacteria, MinD, along with MinE and MinC, rapidly oscillates from one pole of the cell to the other controlling the correct placement of the division septum. MinD binds to the membrane through its amphipathic C-terminal α-helix. This binding, promoted by ATP-induced dimerization, may be further enhanced by a consequent attraction of acidic phospholipids and formation of a stable proteolipid domain. In the context of this hypothesis we studied changes in dynamics of a model membrane caused by MinD binding using membrane-embedded fluorescent probes as reporters. A remarkable increase in membrane viscosity and order upon MinD binding to acidic phospholipids was evident from the pyrene and DPH fluorescence changes. This viscosity increase is cooperative with regards to the concentration of MinD-ATP, but not of the ADP form, indicative of dimerization. Moreover, similar changes in the membrane dynamics were demonstrated in the native inverted cytoplasmic membranes of *E. coli*, with a different depth effect. The mobility of pyrene-labeled phosphatidylglycerol indicated formation of acidic phospholipid-enriched domains in a mixed acidic-zwitterionic membrane at specific MinD/phospholipid ratios. A comparison between MinD from *E. coli* and *Neisseria gonorrhea* is also presented.

1. Introduction

In bacteria, cell division is initiated by formation of the so-called Z-ring in the middle of the cell [1]. The Z-ring is formed of protofilaments of the tubuline-like FtsZ protein, and the assembly occurs on the inner surface of the cytoplasmic membrane. To ensure the correct, central positioning of the division ring, two negative regulation systems are required – Min proteins and nucleoid occlusion [2–4]. While the latter prevents Z-ring formation over the not yet segregated nucleoid occupying the central part of the cell volume, Min proteins protect bacteria from formation of nucleoid-less cells ("minicells") by inhibiting a non-central septum placement. The major protein interfering with FtsZ ring assembly is MinC [5] that targets the membrane-associated FtsZ filaments but is not able to bind to the membrane by itself. Its recruitment to the membrane is mediated by another protein, MinD [6–8]. MinD is therefore responsible for both the MinC localization to and distribution pattern on the membrane. MinD is either permanently positioned on the cell poles as in *B. subtilis* [9] or displays a striking oscillatory behavior together with MinE in e.g. *Escherichia coli* [10]. MinD binds to the membrane by a short conserved C-terminal region, which is not structured upon MinD crystallization [11–13], but is presumed to form an amphipathic helix predicted to align parallel to the membrane surface [14–16]. This membrane targeting sequence (MTS) was shown to mediate a direct attachment between MinD and membrane phospholipids [17], maintaining this function when transplanted to other proteins [15]. While the relatively long MTS of MinD from *B. subtilis* ensures the permanent attachment of the protein to the membrane in these species, the shorter MTS of the *E. coli* MinD requires at least dimerization of MinD for binding and underlies its highly dynamic, oscillatory localization [15]. The molecular mechanism of the membrane affinity modulation is based on the ability of MinD-ATP, but not of MinD-ADP, to form dimers (or even oligomers) with higher affinity to the membrane surface and the antagonistic action of MinE, that stimulates the ATPase activity of membrane-bound MinD, promoting detachment [18,19]. This mechanism, together with spontaneous ADP-ATP exchange, appears sufficient to build numerous physical models explaining the pole-to-pole oscillations of MinD-MinE in *E. coli* [20–25].

Amphipathic helices mediate interaction with the membrane of many other amphitropic proteins [26,27]. Two essential features of this interaction are: i) the binding affinity can be modulated both by protein ligands and by membrane lipid composition, and ii) the binding affects both the protein function and the membrane physical properties. The advantage of these features is in providing a spatially

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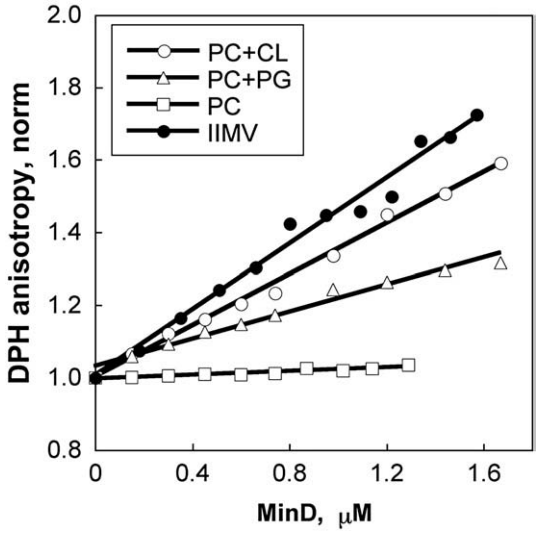


Fig. 1. Dependence of fluorescence anisotropy of DPH on MinD concentration added to liposomes of different phospholipid compositions and to IIMV's. The values are normalized to those in the absence of protein for each type of vesicle. The absolute values of anisotropy in liposomes without MinD were 0.1, 0.08, 0.075 and 0.1 for 100% PC, 40/60% PG/PC, 20/80% CL/PC liposomes and IIMV's, respectively. Membranes (containing 200 μM phospholipids of liposomes and 50 μM of phospholipids for IIMV's) pre-labeled with 5×10^{-7} M DPH (final concentration) were titrated with MinD-ATP to reach the shown concentrations, and fluorescence anisotropy was measured after 10 min equilibration at each concentration.

localized marker for randomly distributed cytoplasmic proteins participating in temporal and spatial regulation mechanisms. *E. coli* MinD displays a preference for anionic phospholipids as shown by in vivo and in vitro studies [15,28]. It was suggested that selection of the cell division site might be regulated by membrane phospholipids composition [28,29]. Existence of membrane heterogeneity or membrane domains is a prerequisite of such regulation. Indications for domains in bacterial membrane were reviewed recently [30]. Most relevant for this consideration are polar localization of cardiolipin (CL) [31,32] explained through an equilibrium mechanism of lipid microphase separation [33], and sequestration of fluorescent acidic and zwitterionic phospholipids into separate pre-existing domains in the bacterial membrane [34]. The combination of the MinD binding dependence on phospholipid composition with the membrane compositional heterogeneity could modulate both spatial and temporal oscillatory characteristics of the protein. On the other hand, putative changes in the membrane physical properties induced by MinD binding may affect other intracellular mechanisms, serving to coordinate between them, e.g. as demonstrated for adjustment of nucleoid morphology and segregation [35].

Part of our working hypothesis is that binding of MinD to the membrane, promoted by the ATP-induced dimerization, is further enhanced by a consequent attraction of acidic phospholipids and formation of a stable proteolipid domain. Furthermore, if an anionic phospholipid domain preexists in a heterogeneous membrane, then high local surface concentration of MinD monomers will encourage protein dimerization and anchoring. The present work tests these assumptions in a model system composed of purified MinD and liposomes of defined composition. Accordingly, the first part of the work is focused on characterization of the changes in the membrane induced by MinD binding as reported by three different membrane-embedded fluorescent probes. In the second part, we compare binding of MinD to the membrane containing a fraction of acidic phospholipids either evenly distributed or concentrated into domains due to phase separation. Moreover, we examine the protein from two different species — rod-shaped *E. coli* and spherical *Neisseria gonorrhoeae* in a quest of generalization of the phenomena under investigation. Actually, MinD's from the two sources are not that different. Having considerably high sequence identity, they both oscillate in *E. coli* and are functionally interchangeable [36]. In view of significant similarity in the phospholipid compositions of the two species [37], we also expect the same behavior of these proteins on the model membrane.

Here we show that binding of MinD causes a remarkable increase in the membrane order and decrease in lateral mobility, with different depth effects in artificial and native (inverted inner membrane vesicles, IIMV's) membranes. We present also indications for domain formation induced by MinD binding at particular fractions of acidic phospholipids in the liposomal membrane.

2. Materials and methods

2.1. Materials

1-Stearoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (SOPC), 1-Stearoyl-2-Oleoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol] (SOPG), CL from heart were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Chloroform was HPLC-grade. 1,6-diphenylhexatriene (DPH), pyrene and 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol, ammonium salt (Py-PG) were obtained from Molecular Probes (Eugene, OR). All other chemicals were analytical grade. Buffers were prepared in deionized water.

2.2. Expression and purification of MinD

His-tagged MinD was overexpressed in *E. coli* strain WM1682 and purified as described in [28] with modifications. Cells from 1 l of culture were suspended in 25 ml cold lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 8 M urea, pH 8.0 and broken by sonication. Cell debris was removed by centrifugation at 10,500 ×*g* for 10 min (4 °C). The supernatant was further centrifuged at 120,000 ×*g* for 90 min (4 °C). Nickel-nitrilotriacetic acid Superflow slurry (Qiagen) was added to the supernatant and the mixture was slowly shaken on ice for 1 h. The slurry was poured into a column and washed 4 times with 8 ml washing buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0 and 0.5 mM phenylmethylsulfonyl fluoride. Nickel-nitrilotriacetic beads were shaken in 12 ml lysis buffer containing 250 mM imidazole for 1 h and beads were removed. The supernatant containing the protein was dialysed three times against a 100 fold volume of dialysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20% glycerol, pH 8.0) containing urea in concentrations 4, 2 and 1 M, and then twice against the same buffer without urea. Protein aggregates were removed by ultracentrifugation (300,000 ×g, 10 min) and the soluble protein was stored at −80 °C. In addition, before each experiment thawed protein was again ultracentrifuged. Protein concentration was determined by the Bradford method and purity was evaluated by SDS-PAGE. A comparison between the protein purified with urea and that obtained as described in [28] has confirmed that their basic membrane-binding behavior, such as phospholipid preferences and nucleotide dependence, was indistinguishable (not shown). We have previously verified that His-tag, connected to the N-terminal of MinD through a linker containing the thrombin cleavage cite, does not interfere with MinD–membrane interaction [28]. Note that the His-tag is neither charged at pH 7.5 nor hydrophobic and is spatially well separated from MinD MTS. Therefore, removal of the His-tag by thrombin cleavage is not necessary for proper interaction of MinD with the membrane and was therefore not performed.

2.3. Preparation of Large Unilamellar Vesicles (LUV)

Phospholipids were dissolved in chloroform and dried to a thin layer film under a gentle steam of nitrogen. Dried phospholipids were hydrated in a 25 mM Tris–HCl (pH 7.5) 50 mM KCl buffer above the phase transition temperature for 1 h and then were vigorously vor-

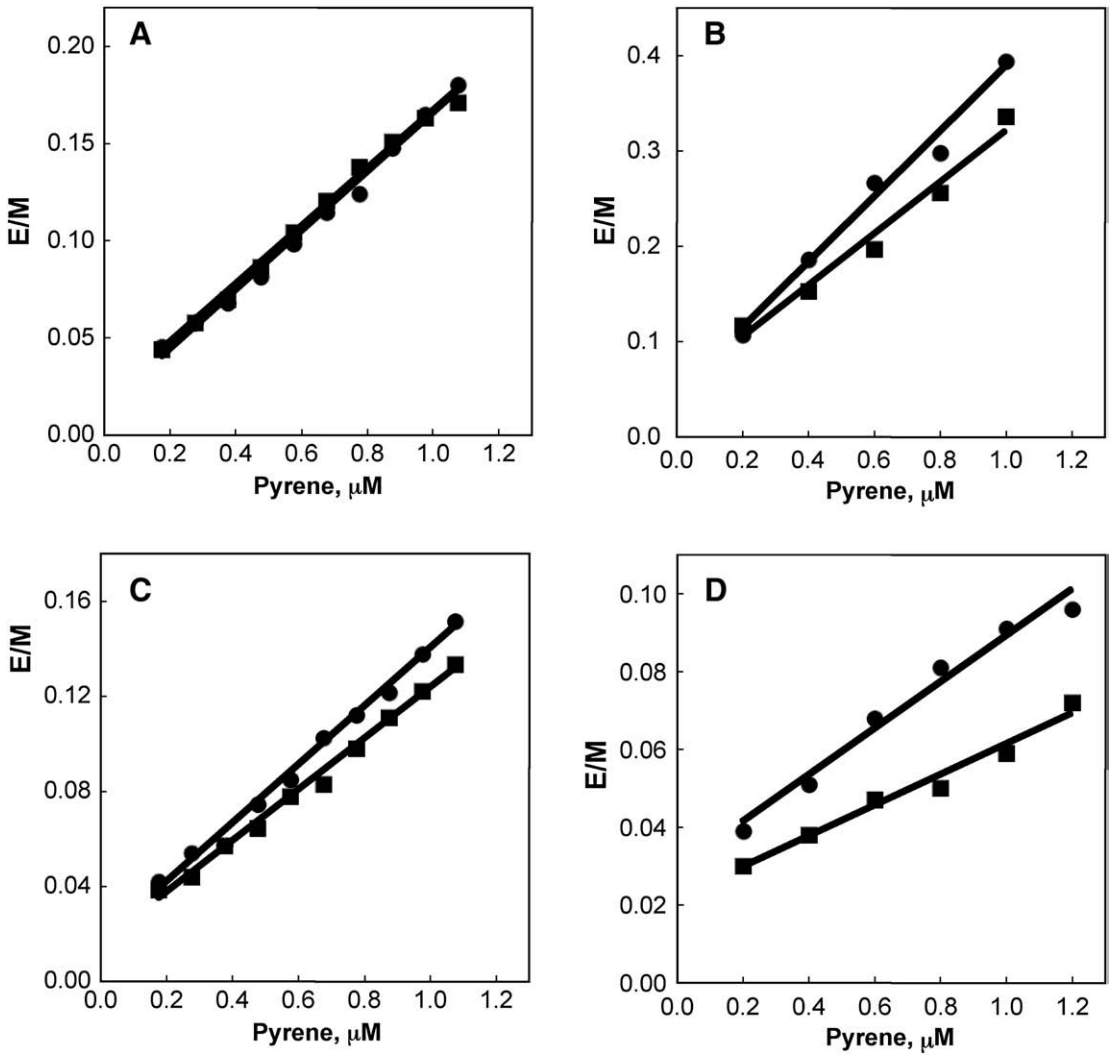


Fig. 2. Dependence of E/M ratio on pyrene concentration in SOPC (A), SOPG (B), and 80/20% SOPC/CL (C) liposomes and in *E. coli* IIMV (D) without (circles) and in the presence of 0.5 μM MinD (squares). 50 μM phospholipids in 25 mM Tris–HCl, pH 7.5, 50 mM KCl, and 5 mM $MgCl_2$ buffer supplemented with 4.5 mM ATP, with or without MinD, were titrated with pyrene within the shown concentration range. Fluorescence spectra were taken at each concentration (see Section 2) and intensities of monomer and excimer were registered at 373 and 470 nm respectively. The slopes of the linear fits are the measure of pyrene excimerization rate.

texed. LUVs were prepared by extruding the suspension through a polycarbonate membrane filter (0.1 μm pore size, 19 mm diameter) 11 times using Avanti Mini-Extruder (Avanti Polar Lipids, Inc.) at room temperature; LUV's were stored at −80 °C. Phospholipids concentration was determined by phosphomolybdate colorimetric assay [38]. To prepare liposomes of desired composition, corresponding volumes of chloroform solutions of individual phospholipids were mixed and processed as described. The phospholipid composition is set as a mole percent of a particular kind (based on 2P per mole for CL) and shown in % throughout the text, figures and legends. Liposomes prepared by the extrusion method were characterized using dynamic light scattering and cryo-electron microscopy. The light scattering measurements yielded a narrow distribution of particles with a mean diameter of about 120 nm. In EM images liposomes appear as unilamelar, with a rare occurrence of inclusion of one small liposome into a larger one. The liposomes are therefore defined as "large unilamelar vesicles" (LUVs) with their membrane almost completely accessible to the protein, according to the phospholipid concentration.

2.4. Preparation of Inverted Inner Membrane Vesicles (IIMV)

IIMVs were prepared according to Futai [39] with minor modifications. BL21(DE3) cells were grown in LB medium at 37 °C overnight, diluted 100 fold and grown to OD_{600}=0.7. Cells were harvested by centrifugation (11,300 ×g for 15 min 4 °C) and washed twice with cold 0.01 M Tris–HCl, pH 8.0. One gram of cells was suspended in 80 ml 0.01 M Tris–HCl, pH 7.4, containing 10 mM $MgCl_2$, 10 μg/ml DNase and 10 μg/ml RNase, 0.375 mM PMSF and 3 mM DTT. Cell suspensions were sonicated on ice for 7 min at 0.04 J. Undisrupted cells were removed by centrifugation at 8000 ×g at 4 °C for 10 min. The supernatant fraction was centrifuged at 100,000 ×g for 30 min. The pellet was washed once and suspended in 0.01 M Tris–HCl, pH 7.4, containing 5 mM $MgCl_2$. Membrane vesicles were stored at −80 °C. Phospholipid concentration in IIMVs was determined by phosphomolybdate colorimetric assay [38] and the amount of IIMVs in reaction mixtures was expressed in phospholipid concentration throughout the text, figures and legends for comparison with that of LUVs.

IIMVs orientation was evaluated from NADH-ferrycyanide oxidoreductase activity [39]. Fluorescence intensity of 1 μM NADH in 0.08M Tris–HCl, pH 7.4, 5 mM KCN and 75 μM potassium ferrycyanide buffer was followed at 340/450 nm at 37 °C. The reaction was started by addition of IIMVs or spheroplasts (3 μg of protein/ml) with or without pretreatment with 1% toluene (37 °C, 10 min). The NADH-ferrycyanide oxidoreductase activity of IIMVs was high comparable with that reported by Futai [39] and insensitive to the toluene treatment, in contrast to spheroplasts where activity was increased about tenfold

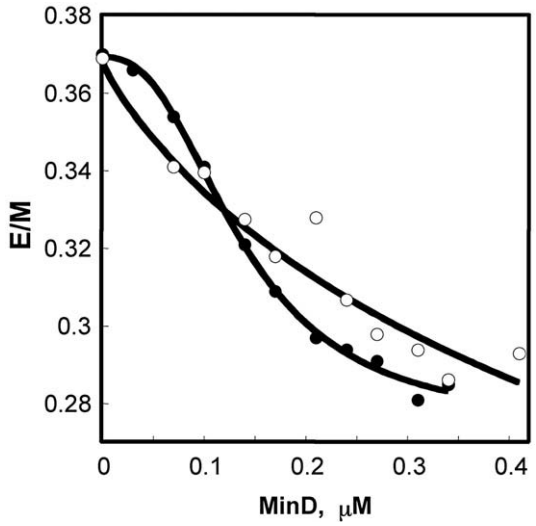


Fig. 3. Dependence of pyrene E/M ratio in SOPG liposomes on MinD concentration. SOPG liposomes (50 μM) pre-labeled with pyrene (1 μM final concentration) were titrated with increasing concentrations of MinD in the presence of 4.5 mM of either ATP (closed circles) or ADP (open circles). Fluorescence spectra of pyrene were recorded after equilibration of the mixture following each protein addition, and the intensities of monomer and excimer were registered (at 373 and 470 nm respectively). The MinD titration curves are fitted with a Hill equation (R of 0.99 and 0.97 for ATP and ADP, respectively).

upon permeabilization with toluene (data not shown). These measurements strongly indicate that the IIMVs are 100% inside-out oriented and may serve as a natural membrane surface for evaluation of membrane interaction with cytoplasmic proteins, such as MinD. Microscopic examination of IIMVs showed a wide distribution of sizes with a typical diameter estimated between 100 to 200 nm. Thus, these membrane preparations are similar to LUVs with respect to the membrane curvature, at least.

2.5. Preparation of spheroplasts

10 ml samples of cells from steady-state growing cultures were centrifuged (5 min, 16,000 ×g, room temperature) and the pelleted cells were resuspended at room temperature in 0.8 ml buffer A (0.01 M Tris, pH 8, 0.1 M NaCl, 20% sucrose) and 0.2 ml buffer B (0.12 M Tris, pH 7.7, 0.05 M EDTA, 0.4 mg/ml lysozyme). In general, within 20–30 min of incubation on ice, ~90% of the cells became spheroplasts.

2.6. Measurement of the membrane dynamics with different fluorescent probes

Steady state fluorescence spectra and anisotropy were measured using Perkin-Elmer LS55 spectrofluorimeter (Perkin-Elmer, Beaconsfield, England) with a cuvette holder thermostated by a circulating water bath (F25ME, JULABO Labortechnik GmbH, Germany). The data were collected and analyzed with dedicated software from Perkin-Elmer. DPH anisotropy in LUV or IIMV suspension containing 50 μM lipid and 0.5 μM DPH (1 mol % in the lipid, added as THF solution to suspension of membranes) was measured at 350 nm excitation and 450 nm emission wavelengths with 2.5/3 nm excitation and emission slits. Additional filters were placed in both the excitation and emission paths to reduce light scattering artifacts. From 20 to 30 readings of the anisotropy were taken after the sample equilibration and the average value was obtained with a typical standard error less than 2%.

The specific feature of pyrene is its ability to form an excited dimer (excimer) in consequence of collision of excited and unexcited monomers, followed by photon emission at a longer wavelength [40–42]. The excimerization reaction depends therefore on pyrene concentration and, most important, on its diffusion rate, reflecting its lateral mobility in the membrane plane and often used to characterize the membrane fluidity. The ratio between fluorescence intensities of excimer to monomer (E/M) provides a direct estimation of pyrene excimerization rate. To refine the diffusion rate, this ratio is measured at various pyrene concentrations and slope in the resulting linear dependence is determined.

Pyrene and Py-PG were excited at 340 nm and fluorescence emission spectra were recorded from 360 to 500 nm with 3/3 nm slits at 100 nm/min scan rate. Fluorescence intensities of monomers and excimers were measured at 373 (377 for Py-PG) and 470 nm, respectively. LUVs and IIMVs suspended in 0.6 ml of 25 mM Tris–HCl (pH 7.5), 50 mM KCl and 5 mM $MgCl_2$ buffer in the cuvette were labeled with pyrene added from 60-μM stock solution in ethanol. The final concentration of ethanol, including titration experiments, never exceeded 2%. Py-PG was introduced into the LUVs membrane at the stage of liposome preparation as a solution in chloroform and mixed with unlabeled phospholipids.

In experiments where the effects of ATP or ADP were tested, 5 mM of nucleotide was added to the buffer to ensure that only the protein concentration and not nucleotide-bound form of MinD is changing during MinD titration experiments. Taking into account the ATPase activity of IIMVs [39], this amount of ATP should be sufficient for the longest titration experiments.

3. Results

3.1. Changes in the membrane order caused by MinD-ATP

The classical and widely used membrane probe DPH reports on the membrane order in the hydrophobic region of phospholipid fatty acid chains [40,43]. Fluorescence anisotropy of DPH increased proportionally to the added MinD-ATP in liposomes composed of SOPC with 40% of PG or 20% of CL, but not in pure PC liposomes (Fig. 1). The latter is not surprising since MinD binding to PC liposomes is very weak at concentrations used herein [28]. The stronger anisotropy changes in CL-containing versus in PG-containing liposomes may be also ascribed to the corresponding higher affinity of the protein to these liposomes. Even more pronounced effect was detected in native membranes (IIMV's) although they contain a large fraction of integral proteins. Notably, the initial (without MinD) order of all tested membranes did not differ significantly (see Fig. 1 legend).

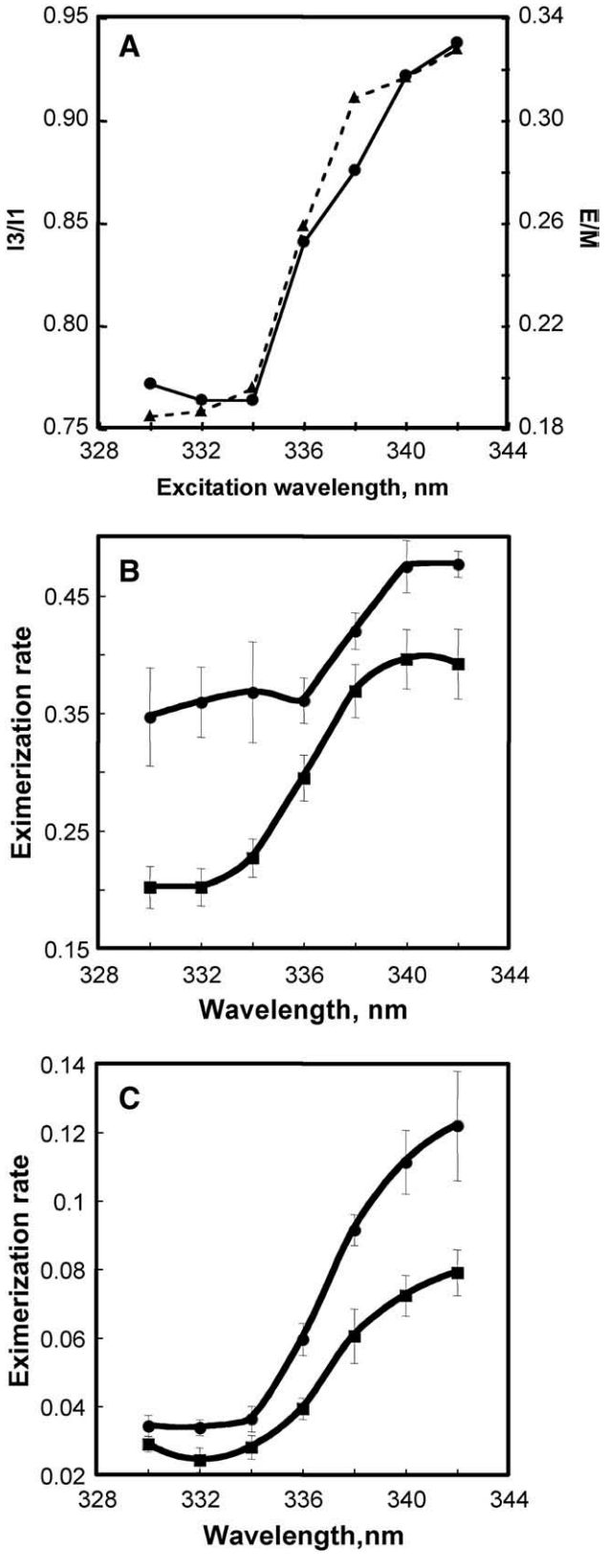
Comparison of MinD from *E. coli* and *N. gonorrhoeae* showed an essential similarity in their effects on the membrane order (data not shown), although the *N. gonorrhoeae* protein increased the membrane order of IIMV's to a somewhat lesser extent (35% instead of 50% at 1 μM concentration of both proteins).

3.2. Changes in the membrane lateral mobility caused by MinD binding

3.2.1. Pyrene excimerization rate

Pyrene, also a highly hydrophobic probe, resides in the fatty acid chain region similar to DPH [40,41], although not as restricted in its location between the membrane surface and hydrophobic core, as will be demonstrated below. Diffusion rates of pyrene measured in liposomes of different composition and in IIMV's revealed differences in their fluidity (Fig. 2), not reflected by DPH-sensed membrane order (see legend to Fig. 1). The apparent diffusion rates of pyrene without added protein were 0.152±0.005, 0.34±0.03, 0.122±0.004 and 0.060 ±0.005 μM^{-1} for PC, PG, PC/CL liposomes and IIMV's, respectively. Incorporation of PG into PC membrane makes it more fluid (see the accompanying paper, Fig. 5), while the native membrane of IIMV's is expected to be the most rigid because of immobilization of phospholipids around numerous integral proteins. This immobilization is not, however, associated with remarkable differences in the membrane packing, or free volume, sensed by DPH. Effects of MinD-

ATP on the membrane fluidity detected by pyrene were essentially consistent with the corresponding results obtained with DPH fluorescence anisotropy: MinD-ATP decreased pyrene diffusion in PG liposomes or PC liposomes containing CL (Fig. 2B and C), with no effect on pure PC membrane (Fig. 2A). Again, the effect on pyrene mobility in IIMV was the strongest – decrease of about 35% (Fig. 2D). MinD from *N. gonorrhoeae* was less effective (data not shown) decreasing fluidity of *E. coli* IIMV by only 15%.



3.2.2. Cooperative changes in the membrane induced by MinD binding

One of the distinctive features of MinD binding to the membrane in vitro is the cooperativity with regards to protein concentration [28,44]. We ascribe this cooperativity to dimerization of MinD on the membrane, manifested also in the FRET experiments [28]. This binding cooperativity should be expressed also in changes of membrane dynamics described above. Indeed, the excimerization rate of pyrene in pure PG liposomes decreased cooperatively with increasing MinD-ATP concentrations with an apparent K_A (MinD) of 0.14±0.01 μM and Hill coefficient of 2.5±0.3 (Fig. 3). In contrast, MinD-ADP displayed no cooperativity and a significantly higher K_A (MinD) of 0.46±0.28 μM. Comparing these results with the binding data from [28], one may mention the much lower range of protein concentrations needed to cause changes in the membrane than those for binding saturation at the same protein/membrane ratio. This suggests that the changes in the membrane, at least those detected by pyrene excimerization, are saturated at lower protein concentration than binding capacity of the membrane. Moreover, at these low concentrations (up to 1 μM MinD) binding of the ATP and ADP forms is hardly distinguishable (Fig. 4 in [28]) consistent with a similar extent in the membrane changes.

3.2.3. Depth effects

The E/M ratio of pyrene fluorescence in the membrane appeared to be strongly dependent on the excitation wavelength in the range from 330 to 342 nm. For example, in pure PG liposomes this ratio increased almost two-fold (Fig. 4A, triangles); similar changes were detected in liposomes of different composition, including zwitterionic and mixed phospholipids (not shown), and also in IIMV (Fig. 4C). Important to note that this phenomenon is specific for membranes in contrast to isotropic organic solvents, where the excimerization of pyrene is completely independent of the excitation wavelength (data not shown). A possible explanation came from a very similar behavior of the peak intensities in the spectrum of pyrene monomer (Fig. 4A, circles). The ratio between vibronic peaks III (383 nm) and I (373 nm) is inversely proportional to environmental polarity [45], serving e.g. as a measure for apparent dielectric constant in protein binding sites [46] or for detection of subtle conformational changes in proteins [47]. In the membrane, the dependence of this ratio on the excitation wavelength presumably reflects selective excitation of pyrene molecules located close to the membrane-water polar interface (excitation range 330–334 nm), or deep in the hydrophobic core of the membrane (excitation above 340 nm). In support of this interpretation, the corresponding ratio in the spectrum of Py-PG, where the position of pyrene moiety is fixed on the end of one of the fatty acids, does not depend on the excitation wavelength (data not shown). Taking such spectral selectivity as depths scan, it follows that the pyrene mobility is higher in the hydrophobic core than in the interface region (Fig. 4A). We used this feature to evaluate the depth effects of MinD binding to the membrane.

In PG liposomes, the overall decrease in excimerization rate induced by MinD, expected from the experiment described above (Fig. 2B), was however much stronger at the blue edge of excitation. In

Fig. 4. Excitation wavelength dependences of pyrene excimerization rate and monomer fluorescence spectrum in the membrane. Ratios of fluorescence intensities at 383 nm (vibronic peak 3, I3, circles) and at 470 nm (excimers, triangles) to that at 373 nm (vibronic peak 1, I1) were measured in SOPG liposomes labeled with 1.5 μM pyrene at different excitation wavelengths (A). Excimerization rate of pyrene incorporated in SOPG LUVs (B) or in IIMVs (C) in the presence of 0.5 μM MinD (squares) or without it (circles) was determined from the pyrene titration (see Section 2). Emission spectra were taken at different excitation wavelengths at each pyrene concentration. The reaction mixture contained 50 μM phospholipids of either LUVs or IIMVs and 5 mM ATP.

other words, MinD binding affects membrane surface in a greater extent than the hydrophobic core. This is consistent with the predicted mode of MinD binding through its amphiphilic C-terminal α-helix, i.e. immersion of the hydrophobic half of the helix into the membrane (as supported by effective quenching of tryptophan by a membrane-embedded nitroxide spin label [17]), with its polar side facing phospholipid head groups [14]. Such immersion is most likely accompanied by an increase in the membrane order mainly in the interface, leaving the fatty acid chains less influenced. In contrast, the effect of MinD on IIMV membrane was inverse: stronger changes in the hydrophobic region and weaker on the surface (Fig. 4C). This discrepancy apparently stems from the large number of integral proteins in the native membrane, immobilizing most of the phospholipids around them. In fact, IIMV, in addition to being more rigid (as mentioned above in *a*), are characterized by a steeper fluidity gradient from the surface to the depth of the membrane: pyrene excimerization rate increased by about 1.5 times from blue to red excitation in PG liposomes without MinD, but more than threefold in IIMVs (compare Fig. 4B and C). It could be that MinD binding to the densely populated surface of IIMV is "transduced" to the membrane depth by integral proteins pressed by the MinD "body" rather than by just MTS insertion. In both types of membranes, MinD binding caused a slight but notable decrease in I3/I1 ratio (data not shown) corresponding to an increase in the polarity of pyrene microenvironment.

3.3. Effects of MinD binding on lateral diffusion and distribution of acidic phospholipids

To obtain more detailed information on the dynamics of boundary phospholipids in the vicinity of the bound MinD and taking into account its head group preferences, we exploited a pyrene derivative of PG in which the pyrene moiety is covalently linked to one of the fatty acids, Py-PG. Fig. 5 displays the behavior of this probe in liposomes of various compositions as a function of MinD concentration. First, in a mostly PC membrane, Py-PG mobility is only slightly decreased by moderate concentrations of MinD (Fig. 5A). Expectedly, this is because of the low affinity of MinD to PC and thus a negligible effect of the protein on PC membrane dynamics (see Figs. 1 and 2) and, on the other hand, too low concentration of Py-PG (5%) to attract MinD. In a pure PG membrane containing the same 5% of Py-PG, MinD addition causes a monotonous and remarkable decrease in the probe mobility (Fig. 5D). This is also consistent with the previous results with pyrene (Figs. 2 and 3) and DPH (Fig. 1) reflecting a general decrease in the membrane fluidity upon the protein binding, including

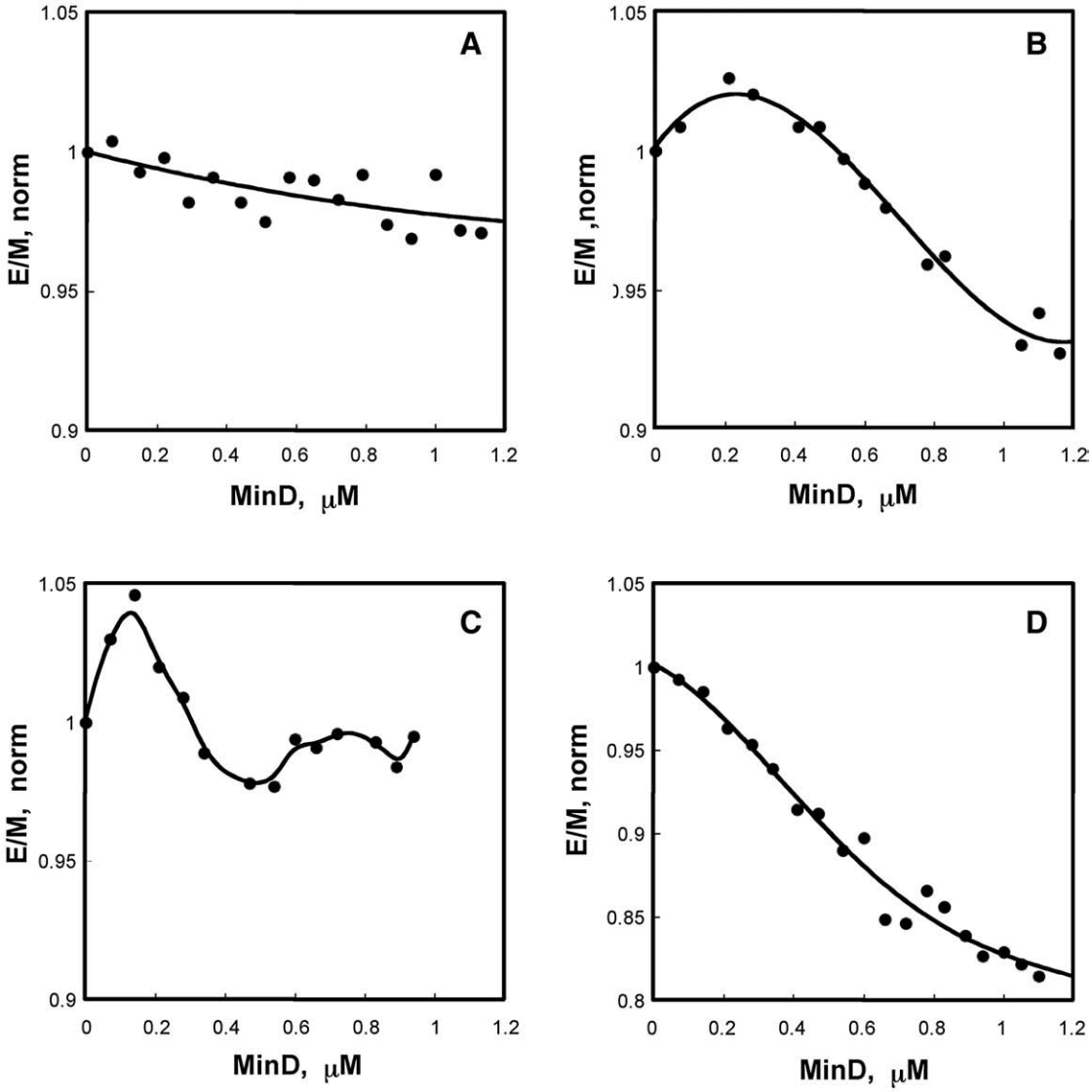


Fig. 5. Excimerization of pyrene-labeled phosphatidylglycerol at increasing amounts of MinD for various fractions of anionic phospholipids. Py-PG/SOPG/SOPC (mole %): (A) 5/0/95, (B) 5/15/80, (C) 5/20/75, (D) 5/95/0. The total phospholipid concentration was kept at 50 μM.

the similar cooperativity of 1.8±0.4 with regards to MinD-ATP and apparent K_A of 0.61±0.10 μM. This apparent association constant is higher than that derived from pyrene excimerization, but still lower than the real protein binding constant [28]. As we have already discussed above in the Section 3.2.2, this may reflect the depth sensitivity of the membrane to MinD binding reported either by free pyrene or by that located at a fixed depth in the Py-PG.

At PG concentrations close to the known physiological ones in the inner membrane of *E. coli* [48], the E/M ratio of Py-PG first increases upon addition of MinD and then becomes lower at increasing concentrations of the protein (Fig. 5B and C). The E/M ratio depends both on the diffusion rate of pyrene (or its derivatives) and on the local concentration of the probe (see Section 2 for explanation). It is unlikely, in the view of the previous results, that protein binding would both enhance and depress Py-PG lateral diffusion at the same time. We therefore adopt the explanation for the same phenomenon observed with another membrane-binding protein [49,50] that is based on an increase in local concentration of Py-PG in a PG cluster formed around bound MinD proteins. Thus, the clusters are progressively formed upon protein binding, but when the protein concentration exceeds the optimal phospholipid/protein ratio, they are presumably dissipated to smaller sizes. Consequently, the high local concentration of Py-PG, exhibited by high E/M ratio, is decreased and the immobilization of the probe around the protein becomes overwhelming in determining the lower excimerization rate. It should be noted that since Py-PG is symmetrically distributed between the leaflets of the membrane bilayer (see Section 2 for the labeling procedure), only half of the probe is exposed to the interaction with the protein. This may be a reason for relatively weak responses to protein binding in experiments shown in Fig. 5.

To distinguish between a general effect of binding of MinD on the dynamic properties of membranes and a more specific, mediated by the direct interaction of the protein with acidic phospholipids, we have compared the response of the Py-PG probe with that of a Py-PC to the protein binding. Mobility of Py-PC is two-fold lower than that of Py-PG at the same probe concentration in the membrane of the same composition (PG:PC=20:80) with a tendency of increasing mobility with decreasing PG content (inverse to Py-PG, see Fig. 5 in the accompanying paper). Changes in Py-PG E/M (Fig. 5) were small but detectable and reproducible, while the changes in Py-PC E/M in low PG content liposomes were hardly distinguishable from the experimental error (not shown). This difference in behavior of two probes is apparently due to their tendency to reside in a similar head group surrounding and, more important, to their attraction to the bound protein. The changes in the Py-PG E/M in the membrane with low PG content are caused by clustering of the probe around the bound MinD, but not merely by a general change in the membrane viscosity (very small in these membranes). The last is much more pronounced in mostly PG membranes at higher MinD concentrations, as detected by all kinds of probes.

4. Discussion

The primary question that we raised in this work, whether binding of MinD to the membrane causes significant changes in its structure and dynamics, has been answered: three different fluorescent probes consistently reported an increase in viscosity and order of artificial and native membranes upon binding of MinD (Figs. 1, 2, 5). In accordance to the known phospholipid preferences for binding [15,28], these structure changes were most dramatic in membranes containing acidic phospholipids. While all results presented herein were obtained with *E. coli* MinD, its homolog from *N. gonorrhoeae* exhibited essentially the same properties regarding its effects on the membrane structure and dynamics, except a more pronounced difference between the ATP and ADP forms, i.e. $MinD_{Ng}$-ADP was less effective (data not shown). *E. coli* IIMV were less affected by $MinD_{Ng}$.

Fluorescence anisotropy of DPH is a measure for the membrane order parameter, i.e. for the packing of fatty acid chains [40]. In fact, small changes of anisotropy reflect much stronger changes in local microviscosity [43] and, therefore, the 50% or more increase reported here (Fig. 1) could be severe enough to cause overall structural rearrangement of liposomes into tubes at higher MinD concentrations [51]. Although MinD concentrations, used herein, are close to in vivo estimates [52,53] and native membranes and liposomes mimicking native phospholipid composition were tested, significantly weaker effects are expected in the cell where the lipid/protein ratio is much higher. However, in the case of pre-existing acidic phospholipid domains, the local changes may be much stronger than the average value reported by the uniformly distributed DPH.

Changes in pyrene diffusion upon MinD binding were less pronounced (Fig. 2), although pyrene was more informative regarding the composition-related differences between liposomes, showing a diffusion rate with an order of PG>CL>PC. Notably, a seeming correlation appeared between the initial membrane fluidity and its sensitivity to protein binding (Fig. 2A–C). Indeed, as it was shown (see the accompanying paper), the enhanced binding of MinD to PC membranes containing increasing fractions of PG may be ascribed both to elevated negative charge and to a higher fluidity of these membranes. However, the strong effect of MinD on IIMVs, exhibiting the lowest fluidity, counters this rule (Fig. 2D).

In experiments described above, IIMVs were added to a sample mixture to reach the same total phospholipid concentration as with LUVs. The phospholipid/protein ratio in the bacterial cytoplasmic membrane is about 25–50 and there might be limiting availability of binding sites for peripheral proteins. According to our estimations, MinD binding capacity of IIMVs is comparable with that of LUVs of a similar phospholipid composition (not shown). Nevertheless, the results obtained with artificial membranes cannot always be extrapolated to the native membranes as was demonstrated by the changes in the depth mobility gradient of pyrene, reported here for the first time. This gradient, being the same in shape, but much steeper in IIMVs than in LUVs (Fig. 4), responded differently to MinD binding to these membranes. Surprisingly, the surface binding of MinD showed effects at greater depth for the native IIMV membrane than for liposomes. The depth effects, however, could not be explained on the basis of the MinD MTS deeper penetration since the amphipathic α-helix can reside only in the interface region [14]. Together with stronger changes in the membrane order of IIMVs reported by DPH (Fig. 1) and in fluidity reported by pyrene (Fig. 2D), one may speculate that in a native membrane, rich in other proteins, the effect of such peripheral protein like MinD may be "amplified" through the surface macromolecular crowding once the protein succeeded to anchor by its MTS.

The cooperative-like character of changes in the membrane dynamics of PG LUV's with regards to MinD concentration (Figs. 3 and 5D) was also expected from the binding properties of the protein, although it was evident with pyrene or Py-PG and not by DPH. A less trivial and a more interesting result is the non-monotonous dependence of Py-PG excimerization rate on the MinD concentration in LUVs containing 20–25% PG (Fig. 5B and C). This behavior, particularly the increase of excimerization, was explained and supported by Monte-Carlo simulations by Hinderliter [50], as formation of membrane domains enriched in the protein and its preferred phospholipid. The driving force for domain formation is thermodynamically favorable clustering of the protein and acidic phospholipids. Py-PG follows the head group partitioning and its excimerization reaches a maximum corresponding to its highest concentration in such domains. A further increase in protein/phospholipid ratio leads to saturation of domains and their dissipation as the limited number of acidic phospholipids becomes distributed among the larger amount of bound protein. Consequently, Py-PG immobilized with the boundary lipids is much less exposed to excimerization. These results show that in a binary mixture of fluid phospholipids MinD may cause formation of domains enriched in

negatively charged lipids. Such a scenario seems less likely to occur in a native bacterial membrane with a very low amount of lipids susceptible for redistribution. Nevertheless, in the second part of this work we show that binding of MinD to a pre-existing acidic phospholipid domain is remarkably enhanced and, moreover, the protein stabilizes such a domain — a phenomenon based on the same mechanism as described above.

Summing up, it is evident from the results presented above that binding of MinD to the membrane can change its dynamic and structural properties and these changes are not negligible, at least in a model system in vitro. Whether and how this phenomenon may be expressed in functional features of the Min system in vivo? If a gradient of MinD concentration from poles to the mid-cell exists in bacteria, as it is seen in microscopic images [10] or predicted from various theoretical models [20–25], then a resultant fluidity gradient might be built up as well. Such a fluidity gradient could influence activity and/or distribution of other integral or peripheral membrane proteins and intracellular systems dependent on the membrane state. This could be a possible explanation for the observed aberrations in the nucleoid segregation pattern in Min-impaired *E. coli* cells [35]. More experimentation is called for to clarify these questions.

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